# MUTANT *E. COLI* STRAINS, AND THEIR USE FOR PRODUCING RECOMBINANT POLYPEPTIDES

The invention concerns certain mutant *E. coli* strains, and their use for performing processes for producing recombinant polypeptides.

Genomic study of higher organisms, micro-organisms, and viruses almost invariably requires, in addition to the cloning of their genes, large-scale production of their products (proteins), so as for example to obtain antibodies or to perform biochemical or crystallographic studies.

From the applications viewpoint, the utilization in the medical field of numerous human peptides and proteins also requires expression of corresponding genes in heterologous organisms.

Although expression systems have been established in various eukaryotic hosts (especially in yeasts, insects and primate cells), the most widely used host for these expression strategies remains the bacteria *Escherichia coli (E. coli)*. The list of proteins of biotechnological or

pharmacological interest that are produced in *E. coli* is extensive; classic examples include human insulin and human growth hormone.

The most well-known expression system in prokaryotes was developed in the USA by the Studier and Richardson groups, during the 1980's (Tabor and Richardson, 1985; Studier and Moffat, 1986). It is based on exploiting the properties of T7 RNA polymerase (namely RNA polymerase encoded by the T7 bacteriophage). That enzyme, which can be expressed in *E. coli* cells without toxicity, recognizes a very specific promoter. Any gene of interest (target gene) may be transcribed very efficiently, upon placing it downstream of this promoter and introducing it into an *E. coli* cell expressing T7 polymerase.

Nevertheless, in terms of expression, the results remain uncertain. Some target genes may be duly overexpressed, whereas others are expressed only moderately or not at all.

Previous work by the inventors revealed that one of the principal causes of these setbacks resides in the specific instability of the m-RNA synthesized by T7 RNA polymerase, which causes a decrease in the number of polypeptides synthesized by messaging (Lopez et al., 1994; Iost and Dreyfus, 1994, 1995). This instability is the

consequence of the high speed of elongation of T7 RNA polymerase (Makarova et al., 1995). Specifically, the elongation speed of T7 polymerase, in contrast to that of bacterial RNA polymerase, is much greater than the translation speed of m-RNA by ribosomes. Nascent m-RNA is therefore exposed over most of its length, and is therefore readily attacked by nucleases, and in *E. coli* especially by the E-type ribonuclease (or RNase E), whose amino acid sequence is described by Casaregola et al. (Casaregola et al., 1992, 1994).

RNase E is an essential enzyme of *E. coli*; it is involved both in the degradation of m-RNA as well as in the maturation of ribosomal RNA (r-RNA). Mutations in the catalytic region (that is, in the N-terminal portion of RNase E) affect these two functions at the same time, and slow down or even arrest the growth of *E. coli* (Cohen and McDowall, 1997).

On the other hand, deletions in the C-terminal portion of RNase E do not affect the viability of *E. coli*. Specifically, by researching revertants of mutations in a protein (MukB) necessary for the segregation of chromosomes after replication, Kido et al. obtained various viable mutations in the *rne* gene, coding RNase E in *E. coli*, which cause synthesis of an RNase E that is truncated in its C-

terminal portion (Kido et al., 1996). These authors concluded from these experiments that the C-terminal portion of RNase E is not essential for viability of *E coli*. They moreover formed the hypothesis that suppression of the *mukB* mutations by truncating of the RNase E, reflects the fact that truncated RNase E is less effective than the wild-type enzyme for degrading *mukB* m-RNA. Thus stabilized, a stronger synthesis of the mutant MukB protein could be achieved, thereby correcting the phenotype associated with the mutation. However, this stabilization of the *mukB* messenger was not demonstrated, and other authors proposed an entirely different interpretation to explain the suppressive effect of the truncating of RNase E on *mukB* mutations (Cohen and McDowall, 1997). These authors postulate in particular a direct interaction between RNase E and MukB. The basis for that idea is the fact that RNase E has a very substantial similarity with eukaryotic myosin (Casaregola et al., 1992: McDowall et al., 1993), which suggests that aside from its own RNase activity, it could, like MukB, play a structural role.

The present invention arises from the demonstration by the inventors of the fact that the truncating of RNase E causes an overall stabilization of cellular m-RNA, considered as a whole, as well as of the majority of individual m-RNAs

that were examined, without significantly impeding the maturation of the r-RNAs (Lopez et al., 1999).

In that regard, the effect of the deletion is very different from that of a mutation in the N-terminal region, such as the *ams* mutation (Ono and Kuwano, 1979), renamed *rne1* (Babitzke and Kushner, 1991), which confers thermosensitive activity to RNase E. For example, at 37°C, this latter mutation causes a moderate increase in the lifespan of the m-RNAs (1.5 times each on average; the lifespan of the m-RNAs is here defined as the time during which they serve as a matrix for protein synthesis (Mudd et al., 1990a)), but it also causes a significant slowdown in maturation of the r-RNAs (estimated by the "Northern" method; see Lopez et al., 1994) and it retards the growth by a factor of 2. On the contrary, deletion of the C-terminal portion of RNase E, especially of amino acids 586 to 1061 of this latter, causes a more significant stabilization of the m-RNA (two times on average), without causing a slowdown in the maturation of the r-RNA and without retarding growth. Thus, in hindsight, it is likely that the lack of growth that was observed with N-terminal mutations of RNase E, is due solely to the inability of the cells to mature r-RNA.

In summary, deletions in the C-terminal portion of RNase E have no effect on the activity of the catalytic

domain, judging from the rapid maturation of the r-RNA. That rapid maturation explains why the cells containing such a deletion are viable. On the other hand, the deletion stabilizes the m-RNA as a whole, perhaps because it inhibits the association of the RNase E with other enzymes within a multi-protein structure, the so-called "degradosome", which might be necessary for degradation of the m-RNA (Carpousis et al., 1994; Miczack et al, 1996; Py et al., 1996; Kido et al., 1996; Cohen and McDowall, 1997). The important point from the perspective of the invention is that, by virtue of these deletions, it is possible to obtain *E. coli* strains having enhanced m-RNA stability, while preserving normal growth.

The inventors have also shown that the stabilization of m-RNA due to the deletion of the C-terminal portion of RNase E, is not uniform, but rather is more pronounced for less stable m-RNA. As is known, this is often the case for the m-RNA of "target" genes in expression systems. The contribution of this m-RNA to the overall protein synthesis is therefore enhanced by the presence of the deletion. *E. coli* strains comprising such a deletion therefore express recombinant exogenous polypeptides with sharply higher yields (in particular about 3 to 25 times higher) with respect to the expression yields of those recombinant polypeptides by *E. coli* strains not comprising

that mutation, especially when the expression of the said recombinant polypeptides is placed under the control of T7 RNA polymerase.

The present invention therefore has as an object to provide novel processes for producing recombinant proteins or polypeptides from *E. coli*, especially those of pharmaceutical or biological interest, at production yields substantially greater than those of the processes described up to now.

The present invention also has as an object to provide novel *E. coli* strains for practicing the above-mentioned processes, as well as methods for preparing such strains.

The present invention has as an object the use of *E. coli* strains whose gene encoding RNase E comprises a mutation such that the enzyme produced upon expression of this mutated gene no longer possesses m-RNA-degrading activity, this mutation not significantly affecting the growth of the said *E. coli* strains, for practicing a process for producing predetermined exogenous recombinant polypeptides (or proteins).

The present invention more particularly concerns the use of *E. coli* strains whose gene coding RNase E comprises a mutation such that the enzyme produced upon expression of this mutated gene preserves the maturation

activity of the r-RNA of the RNase E, but no longer possesses the degradation activity of the m-RNA, for practicing a process for producing predetermined exogenous recombinant polypeptides (or proteins).

The invention more particularly has as an object the above-mentioned utilization of *E. coli* strains as defined above, characterized in that the mutation consists in the substitution or deletion of one or several nucleotides in a region of the gene coding for the C-terminal portion of RNase E.

The invention yet more particularly concerns the above-mentioned utilization of *E. coli* strains as defined above, characterized in that the mutation corresponds to the substitution or to the deletion of one or several nucleotides of the region delimited by the nucleotide situated at position 1935 and the nucleotide situated at position 3623 of the DNA coding RNase E, represented by SEQ ID NO: 1.

Advantageously, the above-mentioned mutation causes modification or deletion of at least one amino acid from the C-terminal portion of RNase E.

To that end, the invention has as an object the above-mentioned utilization of *E. coli* strains as defined above, characterized in that the mutation causes the deletion of at least one, and up to all, of the last 563 amino acids

of the sequence of RNase E represented by SEQ ID NO:2.

The invention more particularly has as an object the above-mentioned utilization of *E. coli* strains as defined above, characterized in that the mutation corresponds to the substitution of the guanine G in position 2196 of SEQ ID NO: 1 by a thymidine T, so as to create a stop codon TAA situated at positions 2196 to 2198 of SEQ ID NO:1.

Advantageously, the above-mentioned mutant *E. coli* strains, used in the context of the invention, contain an exogenous inducible expression system, under the control of which is placed the expression of predetermined recombinant polypeptides, especially the inducible expression system using RNA polymerase of the T7 bacteriophage.

The invention also concerns *E. coli* strains that are transformed such that they contain an exogenous inducible expression system, and whose gene coding RNase E comprises a mutation such that the enzyme produced upon expression of this mutated gene no longer possesses degradation activity for m-RNA, this mutation not significantly affecting growth of the said *E. coli* strains.

The invention also has for an object *E. coli* strains such as described above, transformed such that they contain an exogenous inducible expression system, notably chosen from those described above, and whose gene coding

RNase E comprises a mutation such that the enzyme produced upon expression of this mutated gene preserves the maturation activity for the r-RNA of the RNase E, but no longer possesses the activity of this latter for degradation of m-RNA.

The invention more particularly has as an object *E. coli* strains as described above, characterized in that the inducible expression system uses RNA polymerase coded by the T7 bacteriophage.

The invention also concerns *E. coli* strains as described above, characterized in that the mutation consists in the substitution or deletion of one or several nucleotides from the region of the gene coding for the C-terminal portion of RNase E.

The invention yet more particularly concerns *E. coli* strains as defined above, characterized in that the mutation corresponds to the substitution or deletion of one or several nucleotides from the region delimited by the nucleotide situated at position 1935 and the nucleotide situated at position 3623 of the DNA sequence coding RNase E, represented by SEQ ID NO: 1.

The invention more particularly has for an object mutant *E. coli* strains as defined above, characterized in that the above-mentioned mutation causes modification or

deletion of at least one amino acid of the C-terminal portion of the RNase E expressed by the said strains.

To that end, the invention has as an object *E. coli* strains as defined above, characterized in that the mutation causes deletion of at least one, up to all, of the last 563 amino acids of the sequence of RNase E represented by SEQ ID NO: 2.

The invention more particularly has as an object *E. coli* strains as defined above, characterized in that the mutation corresponds to the substitution of guanine G at position 2196 of SEQ ID NO: 1, by thymidine T, so as to create a stop codon TAA situated at positions 2196 to 2198 of SEQ ID NO: 1.

The invention also has as an object *E. coli* strains as defined above, characterized in that the inducible expression system controls the transcription of a DNA sequence coding one or several predetermined recombinant polypeptides.

The invention also concerns any process for producing predetermined recombinant polypeptides, characterized in that it comprises:

- a step of transforming *E. coli* strains whose gene coding RNase E comprises a mutation such that the enzyme produced upon expression of this mutated gene no longer

possesses degradation activity for m-RNA, this mutation not significantly affecting the growth of the said *E. coli* strains, with a vector, especially a plasmid, containing the nucleotide sequence coding one or several recombinant polypeptides,

- culturing of the transformed *E. coli* strains obtained during the preceding step, for a time sufficient to allow expression of the recombinant polypeptides in the *E. coli* cells,

- and recovery of the recombinant polypeptide or polypeptides produced during the preceding step, if desired after purification of these latter, especially by chromatography, electrophoresis, or selective precipitation.

The invention more particularly has as an object any process for producing predetermined recombinant polypeptides, as defined above, characterized in that it comprises:

- a step of transforming *E. coli* strains as described above, with a vector, especially a plasmid, containing the nucleotide sequence coding one or several recombinant polypeptides, so as to obtain the above-mentioned *E. coli* strains, in which transcription of the said nucleotide sequence coding one or several recombinant polypeptides is placed under the control of an inducible

expression system,

- culturing the transformed *E. coli* strains obtained during the preceding step, and inducing the said expression system, for a time sufficient to permit expression of the recombinant polypeptide or polypeptides in *E. coli* cells, the inducing of the said expression system especially being effected by causing synthesis of T7 RNA polymerase when the said expression system calls for that polymerase; this synthesis may notably be provoked by adding IPTG to the culture medium, or by raising the temperature, following which the gene coding for this RNA polymerase is placed under the control of a promoter regulated by the *lac* repressor (Studier and Moffat, 1986), or under the control of a thermo-inducible promoter (Tabor and Richardson, 1985),

- and recovering the recombinant polypeptide or polypeptides produced during the preceding step.

A general process for obtaining mutant *E. coli* strains as described above, and capable of being used in the context of the present invention, comprises the following steps:

- preparation of a plasmid containing an *rne* gene comprising a mutation as described above, and in which the promoter of the said *rne* gene is suppressed,

- introduction of the plasmid obtained in the

preceding step, into an *E. coli* strain comprising an inducible expression system, as well as a chromosomal mutation in the *rne* gene conferring a particular property to the said *E. coli*, such that the so-called *rne1* mutation (Ono and Kuwano, 1979) rendering the growth of the host thermosensitive, and permitting selecting acquisition of the desired mutation of the *rne* gene on the *E. coli* chromosome,

- culturing the thus-transformed *E. coli* strains, and selecting *E. coli* strains having the particular property mentioned above, namely the clones resulting from the homologous recombination which permits replacing the said chromosomal mutation by the homologous sequence corresponding to the mutated *rne* gene of the said plasmid, especially selection of thermoresistant clones in the case where the chromosome mutation is the said *rne1* mutation,

- elimination of the plasmid from the selected clones, and identification from among these clones of those comprising the above-mentioned mutated *rne* gene, especially by analyzing by electrophoresis the length of the truncated RNase E polypeptide, coded by the above-mentioned mutated *rne* gene, produced by the mutant *E. coli* cells.

The invention will be illustrated to advantage with the aid of the following detailed description of the preparation of a mutant *E. coli* strain according to the invention,

and of its use for producing predetermined polypeptides.

1) Construction of a mutant *rne* gene containing a STOP codon at the 586th codon

This particular position was chosen, as the truncation thus created in the RNase E is formally equivalent to that which results from the spontaneous *smbB131* mutation (here renamed *rne131*) obtained by Kido et al. (1996). This latter is a deletion of 2 nucleotides at the 586th codon, causing a reading frame shift followed by a stop, after a supplementary translation of 32 codons without relation to the normal sequence of RNase E.

To construct such a gene, the G2196 nucleotides of the sequence is substituted by T, creating a TAA codon (stop) at nucleotide 2196-2198 of SEQ ID NO: 1 (this mutation will by convention be designated herein "G2196T").

To create the desired substitution, the wild-type *rne* gene is first subcloned in the pEMBL8$^+$ "phagemid" (Dente et al., 1983). To that end, the entire transcribed sequence of the *rne* gene is amplified from the *E. coli* genome, with the aid of the following primers:

SEQ ID NO: 3: 5'GGG*CTGCAG***TTTCCGTGTCCATCCTTG** 3'

(the sequence in bold corresponds to the nucleotides (nt) 81-98 of SEQ ID NO: 1; the sequence in italics is the recognition sequence of the *Pst*I enzyme), and

SEQ ID NO: 4: 5' GGGA*GATC***TTGATTACTTTGAGCTAA** 3' (the sequence in bold is complementary to nt 3630 to 3647 of SEQ ID NO: 1; the sequence in italics is recognized by the *Bgl*II enzyme).

The amplified fragment is then digested by *Bgl*II and *Pst*I enzymes (these enzymes have no cleavage sites interiorly of the *rne* sequence), and inserted between the *Bam*HI and *Pst*I sites of pEMBL8$^+$ (bearing in mind that the *Bam*HI and *Bgl*II sites may be ligated to one another). It will be noted that the *rne* sequence thus cloned is devoid of its promoter.

Any parasitic transcription issuing from the vector is eliminated by next introducing into the *Pst*I sites of the obtained sequence, and in the same direction as the *rne* gene, the following synthetic fragment:

SEQID NO: 5:

*CTGCAG***ATAGCCCGCCTAATGAGCGGGCTTTTTTTT***CTGCAG*

(the sequence in bold corresponds to a very efficient transcription terminator, of the tryptophan operon (Christie et al., 1981), and the extremities in italics correspond to the sequence recognized by *Pst*I). These precautions guarantee that the *rne* sequence carried by the plasma may not be transcribed from plasmid promoters, and thus that the RNase E may not be synthesized from the

plasmid. The significance of this point will appear later. In the following description, the plasmid thus obtained is named pRNE.

The desired substitution (G ->T) is then introduced into the pRNE plasmid by using the conventional technique of directed mutagenesis described by Kunkel (Kunkel et al., 1987). For that, the pRNE plasmid is introduced into the RZ1032 strain (Hfr KL16PO/45 (*lysA61-62 dut1 ung1 thi1 relA1 supE44 zbd-279::*Tn*10*). The *dut1* and *ung1* mutations present in this strain cause incorporation of deoxyuridine (dU) in place of thymidin (T) in the DNA. The cells are next overinfected by the K07 "helper" M13 phage (Pharmacia), which causes accumulation in the medium of "phages" comprising the sequence of pRNE in the form of a simple strand, with dU in place of T. After deproteinization, this single strand matrix is hybridized with the following synthetic oligonucleotide:

SEQ ID NO: 6: GCGGTGGTTAAGAAACCAAAC

corresponding to the positions 2188 to 2208 of SEQ ID NO:1 (the "T" that is desired to be incorporated in place of G is indicated in bold), then the hybrid is converted to double strand DNA by incubation with Klenow polymerase, T4 ligase, ATP and dNTP (Kunkel et al., 1987). The double strand hybrid is then introduced in XL1, an *E. coli* strain

conventionally used for cloning (Stratagene). This strain is native for the *dut* and *ung* genes, and consequently the initial strand comprising dU in place of T will be degraded. The vast majority of the resulting XL1 colonies thus comprise the desired mutation in the pRNE plasmid.

By choosing four candidates, it is assured that the desired mutation is clearly present, and that the plasmid does not comprise any others. In that regard, appropriate oligonucleotide primers are used to determine the sequence of the *Afl*II-*Nru*I region (nt 1931-2345 of SEQ ID NO: 1), and only those candidates comprising in this region the single desired mutation are selected, to the exclusion of any other modification. The *Afl*II-*Nru*I fragment issuing from such a candidate is then isolated and the *Afl*II-*Nru*I fragment of the initial (non-mutagenisized) pRNE plasma is substituted therein. There is thus obtained a plasmid comprising the desired mutation; this plasmid is designated hereinafter as pRNE-STOP.

2) Introduction of the mutation creating a stop at codon 586 of RNase E, on the BL21(DE3) chromosome, a strain expressing the RNA polymerase of the T7 bacteriophage.

*General principle*. The desired mutation (G2196T) produces no phenotype change relative to the wild-type gene. To introduce it onto the chromosome, it is therefore

necessary to proceed in two steps: first, a false-direction mutation is introduced into RNase E at codon 66 (the mutation designated *ams*, or *rne1*; Ono and Kuwano, 1979). This mutation corresponds to the G636A transition, according to the numbering of SEQ ID NO: 1 (McDowall et al., 1993). It decreases the thermal stability of the RNase E, impeding high temperature growth.

Next, the pRNE-stop plasmid is introduced into the resulting strain, and the cells that are able to grow anew at high temperature are selected. It will be recalled that the *rne-stop* gene carried by the plasma, being non-transcribed, does not lead to the synthesis of a functional RNase E. In any event, by virtue of a homologous double recombination, the plasmid can carry to the chromosomal *rne* gene the wild-type sequence at position 636 (A636G mutation), reestablishing at the same time the high temperature growth. The homologous region between the plasmid and the chromosomal *rne* region extending over about 1500 nt downstream of the G2196T mutation carried by the plasma, this latter mutation has a strong likelihood of being transferred onto the chromosome at the same time as a A636G. The plasmid is then eliminated; the result is a strain comprising the sole mutation G2196T in the chromosomal gene of RNase E.

*Preparation of an rne1 (ams) derivative of the*

*BL21(DE3) strain.* BL21(DE3) is the typical host for bacterial expression systems based on transcription of heterologous genes by T7 polymerase (Studier & Moffat, 1986). Techniques permitting introduction of the *rne* mutation in any desired genetic context have been described, especially for BL21(DE3) and its derivatives (Mudd et al., 1990b; Iost & Dreyfus, 1995). Briefly, one starts from a bacterial strain (CH1828), comprising the *ams/rne1* mutation as well as a tetracycline resistance gene inserted in a chromosomal locus (*zce-726*) situated a short distance from the *rne* gene. Using the conventional technique known as P1 bacteriophage transduction (Silhavy et al., 1984), a long region of the CH1828 chromosome of several tens of thousands of nucleotides and surrounding the *zce-726* locus, is transferred into BL21(DE3), by selecting acquisition of resistance to tetracycline ($Tet^R$). A high proportion (about 50%) of these $Tet^R$ clones also display thermosensitive growth, which indicates that they have also received the *rne1* allele. The resulting strain is named BL21(DE3)*rne1*.

*Introduction of the G2196T mutation onto the BL21(DE3) chromosome.* BL21(DE3)*rne1* is transformed with the pRNE-stop plasmid (and, as a control, with the initial plasmid pEMBL8$^+$), then, after growth in complete liquid medium (LB medium; (Miller, 1972)) at 30°C, about $10^5$

bacteria are spread out on Petri dishes containing the same medium in agarose, and then incubated at 42°C. For the control bacteria, no growth was observed after 24 hours (the *ams/rne1* mutation does not spontaneously reverse). In contrast, the bacteria transformed with pRNE-stop show a large number of thermoresistant clones, arising from reversion of the chromosomal *rne1* mutation by virtue of the wild-type sequence carried by the plasmid. A dozen of these thermoresistant clones are then chosen, and the plasmid is eliminated from these candidates by cultivating without ampicillin for about 20 generations in LB liquid medium (42°C). Ampicillin is necessary for maintaining plasmids derived from pEMBL8+; in its absence, the plasmid segregates quite readily (Dreyfus, 1988). After re-isolation on LB/agarose medium of the candidates thus treated, loss of the plasmid was verified by testing that the individual colonies could no longer grow in the presence of ampicillin.

It remains to identify those of the thermoresistant revertants - the majority - which, at the same time as the wild-type sequence at position 636, have also acquired the G2196T mutation. This proceeds in two steps. First, the candidates are re-isolated on agarose minimum medium (we use M63B1 medium with glycerol as a carbon source; (Miller, 1972)); by way of control, the BL21(DE3) initial strain and

the BL21(DE3) *rne1* thermosensitive mutant are also spread out on the same dishes. These are then incubated at 43°C. The G2196T mutation causes a slight slowdown of growth in these extreme conditions; the studied recombinants therefore lead to smaller colonies than the wild-type BL21(DE3) cells, which permits an initial screening for the study of these recombinants. The final test resorts to direct determination of the size of the RNase E polypeptide, using the "Western" immunological technique (Sambrook et al., 1989). Briefly, the various candidates (as well as the two controls mentioned above) are grown in LB liquid medium. When the optical density of the cultures at 600 nm reaches 0.5, the cells are harvested. They are then re-suspended in a phosphate buffer and lysed by sonication. After elimination of debris, the proteins in the cellular extract are determined (Bradford, 1976), and then 20 µg of the protein mixture is subjected to electrophoresis according to the Laemmli technique (Laemmli, 1970), by using a 7.5% polyacrylamide gel. This technique allows separating proteins according to their size. After electrophoresis, the protein mixture is electro-transferred onto a nitrocellulose membrane. The membrane is then saturated with non-specific proteins, and then incubated with a 1/10,000 dilution of a polyclonal antibody against RNase E, raised in rabbits. The regions of the membrane having fixed

the anti-RNase E antibody are detected, by incubating this latter with a goat antibody raised against rabbit IgG, and coupled to peroxidase enzyme. The presence of the peroxidase on the membrane is itself revealed by the electrochemiluminescence (ECL) technique, using a kit sold by Amersham. This technique permits determining to what position has migrated the RNase E polypeptide synthesized by each of the candidates, and thus the size of this polypeptide. In particular, the reduction in size occasioned by the G2196T mutation is immediately visible in these tests. More than half of the thermoresistant candidates obtained in this experiment possess the desired mutation.

*Particular case of the rne131 mutation.*

The protocol described above was also used to introduce onto the BL21(DE3) chromosome spontaneous mutations (such as *rne131*) isolated by Kido et al., and also leading to the synthesis of a truncated RNase E. However, these mutations being from the outset localized on the chromosome, the protocol thereof was simplified.

The BZ31 strain (Kido et al., 1996) carries the *rne131* mutation. By virtue of the transduction by P1 bacteriophage (Silhavy et al., 1984; see above), there is transposed in BL21(DE3)*rne1* the region of the BZ31 chromosome surrounding the *rne* locus by selecting transductance capable

of growing at 42°C. Next, it is verified that these clones sufficiently synthesize a truncated RNase E polypeptide by using the "Western" technique described above. All of the tested candidates (6/6, or 100%) acquired the desired modification. Incidentally, it was also observed, as was expected in view of the experiment for constructing BL21(DE3)*rne1*, that 50% of the thermoresistant transductants also acquired the wild-type *zce-726* locus, and therefore once again became sensitive to tetracycline ($Tet^S$). In the following, a $Tet^S$ candidate called BL21(DE3)*rne131* was chosen.

3) Utilization of the BL21(DE3)*rneG2196T* or BL21(DE3)*rne131* strains for efficient gene expression, controlled by the T7 promoter.

*Principle*. The *rneG2196T* or *rne131* mutations cause overall stabilization of m-RNA by a factor of about 2. However, this stabilization is not uniform for all of the m-RNAs. In particular, no doubt because of the particular properties of T7 RNA polymerase (which enzyme has an elongation speed much higher than that of the ribosomes which translate the message; see above), the m-RNA synthesized by this enzyme seems as advantageously stabilized as the majority of cellular m-RNA. It therefore results that the proportion of the total proteins constituted by the products of these particular m-RNAs, is increased when a mutation such

as *rneG2196T* or *rne131* is present in the cell. This observation is the basis of the present invention. Several examples are given below.

*Quantitative evaluation of the invention: the lacZ gene as a model system.* Several years ago, the inventors described the construction of a BL21(DE3) derivative, called ENS134, which comprises a copy of the *lacZ* gene inserted in the *malA* region of the chromosome (Iost & Dreyfus, 1995; Lopez et al., 1994). This gene, which codes for an *E. coli* enzyme - β-galactosidase - whose expression is especially easy to quantify (Miller, 1972), is placed under the control of the T7 promoter. It is followed by a gene coding for a particular t-RNA, $t\text{-}RNA^{Arg5}$ of *E. coli*, whose expression provides a convenient measure of the level of transcription (Lopez et al., 1994). This well-defined system permits particularly reproducible measurements of the stability of a particular m-RNA synthesized by T7 polymerase, as well as of the yield of the corresponding polypeptide. By that test, the *rne131* mutation was introduced in ENS134 as described above for BL21(DE3). We grew ENS134 cells, or the derivative thereof carrying the mutation, at 37°C. As regards the culture medium, we used a rich synthetic medium or a minimum medium (Neidhart et al., 1974), in the presence of IPTG (isopropyl β-D-thiogalactopyranoside; this is an inductor

whose presence is necessary for the synthesis of T7 polymerase in BL21(DE3); Studier & Moffat, 1986). The cells are harvested in exponential phase, then lysed by sonication, whereupon β-galactosidase is determined in the cellular extract, either by measuring enzymatic activity, or by examining the abundance of the β-galactosidase polypeptide by electrophoresis according to Laemmli. It is observed that the presence of the mutation enhances the expression of β-galactosidase by a factor of about 25 in rich medium, or 80 in acetate minimum medium, without affecting the level of transcription of the gene. This result, obtained with a model system, illustrates the possibilities of the invention.

*Expression of cloned eukaryotic genes in E. coli.*

In expression systems based on the properties of T7 polymerase, the gene to be expressed - generally a eukaryotic gene - is fused downstream of the T7 promoter and a ribosome fixation site (RBS) permitting the translation in *E. coli*. The construct is inserted into a multicopy plasmid derived from pBR322, designated pET, and placed in the BL21(DE3) strain or in one of its derivatives (Dubendorff & Studier, 1991; Studier & Moffat, 1986; Studier et al., 1990.) As above, expression of the gene to be expressed - the "target" gene - is initiated by addition of the IPTG inducer to the

culture medium, which causes synthesis of T7 polymerase. However, in contrast to the model system described above, the induction by IPTG in this case may be only transitory, as the transcription of the gene from the T7 promoter is so active that it kills the cells when this promoter is present on a multicopied plasmid.

There were introduced into BL21(DE3), and into BL21(DE3)*rne131* or BL21(DE3)*rneG2196T*, pET plasmids comprising a certain number of eukaryotic genes, namely the *Krox-20* gene implicated in the precocious development of mice (Vesque & Charnay, 1992), the *engrailed-2* gene implicated in the morphogenesis of chicken embryo (Logan et al., 1992), and the gene coding for HTLV1 protease, a human retrovirus (Malik et al., 1988). The cells are caused to grow to a $DO_{600}$ of about 0.5, then IPTG is added. The cells were harvested four hours later. There was thus obtained a cellular extract that is analyzed by electrophoresis as described above. In the three cases, detection of the product of the "target" gene is performed by the "Western" technique (polyclonal and monoclonal antibodies raised against *Krox-20* and *engrailed-2*, respectively, have been described: (Patel et al., 1989; Vesque and Charnay, 1992)). It could be observed that the product of the "target" gene is three to ten times more abundant when the host is BL21(DE3)*rne131* rather than

BL21(DE3).

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